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β-arrestins negatively control human adrenomedullin type 1-receptor internalization

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ABSTRACT

Adrenomedullin (AM) is a potent hypotensive peptide that exerts a powerful variety of protective effects against multiorgan damage through the AM type 1 receptor (AM_1 receptor), which consists of the calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 2 (RAMP2). Two β-arrestin (β-arr) isoforms, β-arr-1 and β-arr-2, play a central role in the agonist-induced internalization of many receptors for receptor resensitization. Notably, β-arr-biased agonists are now being tested in phase II clinical trials, targeting acute pain and acute heart failure. Here, we examined the effects of β-arr-1 and β-arr-2 on human AM_1 receptor internalization. We constructed a V5-tagged chimera in which the cytoplasmic C-terminal tail (C-tail) of CLR was replaced with that of the β_2-adrenergic receptor (β_2-AR), and it was transiently transfected into HEK-293 cells that stably expressed RAMP2. The cell-surface expression and internalization of the wild-type or chimeric receptor were quantified by flow cytometric analysis. The [^{125}I]AM binding and the AM-induced cAMP production of these receptors were also determined. Surprisingly, the coexpression of β-arr-1 or -2 resulted in significant decreases in AM_1 receptor internalization without affecting AM binding and signaling prior to receptor internalization. Dominant-negative (DN) β-arr-1 or -2 also significantly decreased AM-induced AM_1 receptor internalization. In contrast, the AM-induced internalization of the chimeric AM_1 receptor was markedly augmented by the cotransfection of β-arr-1 or -2 and significantly reduced by the coexpression of DN-β-arr-1 or -2. These results were consistent with those seen for β_2-AR. Thus, both β-arrs negatively control AM_1 receptor internalization, which depends on the C-tail of CLR.

1. Introduction

β-arrestins (β-arrs) (non-visual arrestins) are best known for their ability to desensitize G protein-coupled receptors (GPCRs); in 1990, they were first identified as intracellular adapter proteins that can desensitize the β_2-adrenergic receptor (β_2-AR), a family A GPCR [1]. There are two β-arr isoforms, β-arr-1 and β-arr-2, and they share approximately 80% sequence identity [2]. It is now clearly established that these β-arrs are capable of interacting with distinct signaling partners, thereby regulating a variety of downstream pathways of GPCRs [3]. Notably, some agonists can selectively mediate signaling through β-arrs (β-arr-biased signaling) while blocking signaling through G proteins (G protein-biased signaling) [4–6]. To date, most of the attention in the field of GPCR pharmacology has concentrated on using β-arr-biased agonists in the development of new drugs; these agonists are now being evaluated in phase II clinical trials (μ-opioid receptor for acute pain [7] and angiotensin II type 1 receptor for acute heart failure [8]).

GPCR desensitization is primarily mediated through receptor

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phosphorylation by second messenger-dependent kinases (heterologous desensitization) and GPCR kinases (GRKs) (homologous desensitization) [9,10]. GRKs phosphorylate the intracellular regions of GPCRs activated by agonists, after which β-arrs bind to the phosphorylated sites. Thereafter, β-arrs play a central role in desensitization, internalization and the signaling of a number of GPCRs [9,10]. GPCR internalization is required for the resensitization of the receptor.

The calcitonin receptor-like receptor (CLR) is a family B GPCR that can appear at the cell surface, but only when it is coexpressed with three receptor activity-modifying proteins (RAMPs) that possess a single membrane-spanning domain [11,12]. RAMPs have at least eleven GPCR partners, including many family B GPCRs [13]. The combination of CLR and RAMP2 or RAMP3 forms two functional (AM) receptors, AM_1 and AM_2 receptors, respectively. AM is a potent hypotensive peptide that has been demonstrated to powerfully exert a variety of protective effects against multiorgan damage [14,15]. Similar to β-arrs [2], both AM receptors are ubiquitously expressed in human tissues [16,17]. However, only AM_1 receptors are critical for fetal cardiovascular development and protect central and vascular integrity and homeostasis [18–21].

The cytoplasmic C-terminal tail (C-tail) of human (h)CLR contains 12 Ser/Thr residues that are potential phosphorylation sites [22]. We have demonstrated that in the presence of hRAMP2, the hCLR C-tail is essential for interactions with Gs, Gq and Gi [22,23] and for the AM-induced internalization of the AM_1 receptor. The receptor internalization is dependent on GRKs 2, 3 and 4 among the five non-visual GRKs 2 through 6 [22]. We have also demonstrated the marked inhibitory effects of GRKs 4 and 5 on the cell surface expression and signaling of the AM_1 receptor [24]. However, little is known about the relationship between the overexpression of β-arrs or their dominant-negative proteins and the agonist-mediated internalization of RAMP-interacting family B GPCRs, in contrast to that of family A GPCRs. To address this issue, we investigated these effects by using a CLR C-tail chimera (CLR/β_2-AR), in which the C-tail of CLR was substituted with that of β_2-AR, a family A GPCR that is unable to interact with RAMPs, in HEK-293 cells that stably expressed hRAMP2, which enables CLR to function as an AM_1 receptor.

2. Materials and methods

2.1. Reagents and antibodies

The [^{125}I]hAM (specific activity 2 μCi/pmol) was produced in our laboratory [22]. The human AM was kindly donated by Shionogi & Co. (Osaka, Japan). The FITC-conjugated mouse *anti*-V5 monoclonal antibody (*anti*-V5-FITC antibody) was purchased from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers. The plasmid DNAs for each of the two β-arrs (β-arr-1 and β-arr-2) and their dominant-negative constructs (β-arr-1-V53D and β-arr-2 (284–409)) were kind gifts from Dr. Yumiko Saito [25].

2.2. Expression constructs

Double V5-tagged hCLR (V5-hCLR) [26] was cloned into the expression vector pCAGGS/Neo [23], yielding pCAGGS-V5-hCLR. The human β_2-adrenergic receptor (β_2-AR, GenBank accession no. NM_000024) was cloned from cDNA obtained from a human cerebellum (Clontech) by PCR using the appropriate primers. A chimera, V5-CLR/β_2-AR (Fig. 3), was generated by replacing the CLR C-tail with the corresponding sequence from β_2-AR using a restriction site (*Avi* II site) [27] that was situated in the middle of the putative eighth helix [27]. Indeed, the restriction site was newly introduced without altering the amino acid sequence of the receptor because the C-tails of CLR and β_2-AR do not contain any of the common sites. The aforementioned PCR products were all sequenced using an Applied Biosystems 310 Genetic Analyzer (Foster City, CA, USA).

2.3. Cell culture and DNA transfection

Human embryonic kidney (HEK)-293 cells stably expressing hRAMP2 [22] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 100 μg/ml hygromycin B (Wako, Osaka, Japan) at 37 °C under a humidified atmosphere of 95% air/5% CO_2.

The transient transfection of HEK-293 cells stably expressing hRAMP2 was accomplished using the Lipofectamine™ and Plus™ regents (Invitrogen). Briefly, the cells were seeded into 12-well (for flow cytometric analysis) or 24-well plates (for binding and cAMP assays). Upon reaching 70–80% confluency, the cells were transfected with an empty vector (pCAGGS/Neo) (*Mock*) or wild-type (WT) or chimeric V5-tagged constructs. DNA complexing was accomplished by incubating the cells for 4 h in OptiMEM 1 medium containing the plasmid DNAs and the Plus and Lipofectamine regents (see Ref. [27]). The ratio of the transfection amounts of V5-CLR/β_2-AR to each of the β-arrs or their dominant-negative constructs was 1:2; the total transfection amounts were equal because the empty vector DNAs for all β-arr constructs tested were added in accordance with the reduced transfection amounts of the β-arr constructs. All of the following experiments were performed 36–48 h after transfection.

2.4. Flow cytometric analysis

Flow cytometry was used to assess the cell surface expression and AM-induced internalization of V5-CLR or V5-CLR/β_2-AR that was expressed in HEK-293 cells stably expressing RAMP2. Following the co-transfection of the indicated V5-tagged cDNAs into the hRAMP2-expressing cells in 12-well plates, the cells were exposed to 100 nM hAM in prewarmed serum-free DMEM containing 20 mM Hepes and 0.5% bovine serum albumin for 60 min at 37 °C. Receptor internalization was halted by adding ice-cold PBS, after which the cells were washed once with PBS and were then non-enzymatically harvested with ice-cold FACS buffer [23]. After centrifugation, the cells were resuspended in FACS buffer and labeled with the *anti*-V5 FITC antibody (diluted to 1:1000 in FACS buffer) for 2 h at 4 °C in the dark. Following two successive washes, the cells were subjected to flow cytometry with an EPICS XL flow cytometer (Beckman Coulter). The cell surface expression frequency of each V5-tagged receptor (% of cells) was analyzed using the EXPO 2 software (Beckman Coulter) [23]. In this text, the "cell surface expression of the receptors" means "the percentage of FITC-positive cells (V5-CLR- or V5-CLR/β2-AR-expressed cells)" in the 10,000 cells collected during flow cytometry.

2.5. Radioligand binding assays

To assess the whole-cell radioligand binding, transfected HEK-293 cells in 24-well plates were washed once with prewarmed 0.1% BSA/PBS to reduce the non-specific binding of AM. Subsequently, the remaining adherent cells were washed with ice-cold PBS. The cells were then incubated with [^{125}I]hAM (40 pM) for 4 h at 4 °C in the absence (for total binding) or presence (for nonspecific binding) of 1 μM unlabeled hAM in modified Krebs-Ringers-HEPES medium [23]. After washing once with ice-cold PBS, the cells were solubilized with 0.5 ml of 0.5 M NaOH, and the associated cellular

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